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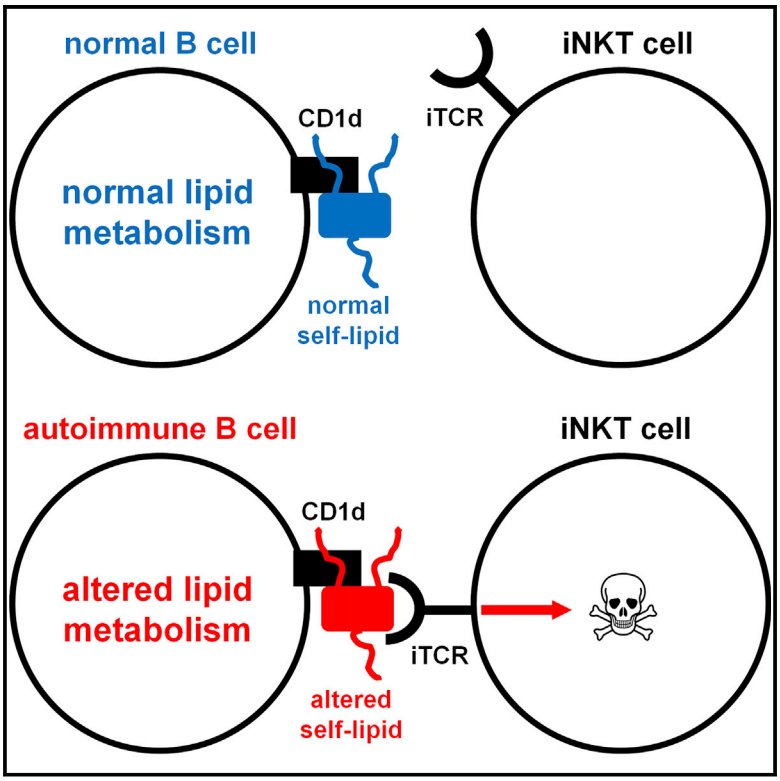
Aberrant Presentation of Self-Lipids by Autoimmune B Cells Depletes Peripheral iNKT Cells

Graphical Abstract



Highlights

iNKT cells are numerically reduced in mice with B cell-mediated autoimmunity

Residual iNKT cells in autoimmune mice are hyperactivated

Autoimmune B cells present CD1d-restricted self-lipids that deplete iNKT cells

Autoimmune B cells have altered lipidome with aberrant expression of certain lipids

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In Brief

Invariant natural killer T (iNKT) cells help B cell antibody production, but how B cells reciprocally modulate iNKT cell responses is less clear. Tan et al. now identify inappropriate presentation of CD1d-restricted self-lipids by autoimmune B cells as a primary mechanism leading to iNKT cell hyperactivation, proliferation, and apoptosis in autoimmune mice. Autoimmune B cells are shown to have an altered lipidome, suggesting that this is linked to iNKT homeostasis.

Tan et al., 2014, Cell Reports 9, 24–31
October 9, 2014 ©2014 The Authors
http://dx.doi.org/10.1016/j.celrep.2014.08.043

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Aberrant Presentation of Self-Lipids by Autoimmune B Cells Depletes Peripheral iNKT Cells

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http://dx.doi.org/10.1016/j.celrep.2014.08.043

SUMMARY

Invariant natural killer T (iNKT) cells provide cognate help via CD1d to lipid antigen-presenting B cells for antibody production, but whether B cells reciprocally regulate iNKT cells remains largely unexplored. Here, we found peripheral, but not thymic, iNKT cells to be numerically reduced in autoimmune mice lacking *Fas* specifically in B cells. The residual iNKT cells were antigenically overstimulated, had altered cytokine production, and manifested enhanced proliferation and apoptosis. B cell-specific ablation of CD1d ameliorated these iNKT defects, suggesting that inappropriate presentation of CD1d-restricted self-lipids by autoimmune B cell-depleted peripheral iNKT cells. CD1d^{+} autoimmune B cells have reduced α-galactosidase A expression and, as revealed by lipidomic profiling, altered lipidome with aberrant accumulation of certain self-lipids and reduction of others. These findings unveil a critical link between autoimmunity, B cell lipidome, and the maintenance of peripheral iNKT cells and highlight an essential homeostatic function of B cells beyond antibody production.

INTRODUCTION

Invariant natural killer T (iNKT) cells are specialized T cells bearing NK lineage receptors and T cell receptors (TCRs) comprising an invariant Vα-Jα chain preferentially associated with a limited set of Vβ chains that recognize glycolipid antigens presented by CD1d (Bendelac et al., 2007). These antigens include glycosphingolipids such as α-galactosylceramide (α-GalCer) (Kawano et al., 1997) and isoglobotrihexosylceramide (Zhou et al., 2004) and glycolipids from Gram-negative (Kinjo et al., 2005; Mattner et al., 2005) and Gram-positive (Kinjo et al., 2011) bacteria. Recognition of foreign and self-lipids is critical to the immunomodulatory functions of iNKT cells (Godfrey and Kronenberg, 2004; Taniguchi et al., 2003), which include the promotion of B cell antibody production, plasma cell generation, and memory B cell recall responses (Barral et al., 2008; Galli et al., 2007; Leadbetter et al., 2008; Tonti et al., 2009).

How B cells in turn modulate iNKT cell responses is far less known. Human B cells capture exogenous lipid antigens using apolipoprotein E and low-density lipoprotein receptor, leading to enhanced lipid presentation via CD1d to iNKT cells (Allan et al., 2009). Splenic marginal zone (MZ) B cells also trigger iNKT cells to proliferate and produce interleukin-4 (IL-4) and IL-13 (Zietara et al., 2011) and preferentially provide cytokine signals to dendritic cells for optimal iNKT activation (Bialecki et al., 2009). Recently, B cells from systemic lupus erythematosus (SLE) patients were shown to activate iNKT cells poorly compared with those from healthy donors and resulted in reduced iNKT cell frequencies in their peripheral blood (Bosma et al., 2012). A similar decrease in iNKT cells was observed in patients with type 1 diabetes (Kukreja et al., 2002) and inflammatory arthritis (Tudhope et al., 2010). However, it was not clear if iNKT reduction resulted from genetic mutations affecting iNKT cells intrinsically or extrinsically via antigen-presenting cells or both. Moreover, studies with human blood leukocytes might not reflect how B and iNKT cells interact in peripheral organs.

Here, we found peripheral iNKT cells to be hyperactivated but numerically reduced in mice with FAS (CD95, Apo-1) ablated in B cells (Hao et al., 2008). Further analysis revealed autoimmune B cells have altered lipidome and disrupted iNKT cell homeostasis in a CD1d-dependent manner.

RESULTS AND DISCUSSION

iNKT cells were reported to be numerically reduced in germline MRL-*lpr* (*Fas* mutant) mice and autoimmune patients (Bosma et al., 2012; Yang et al., 2003), although it was not clear why

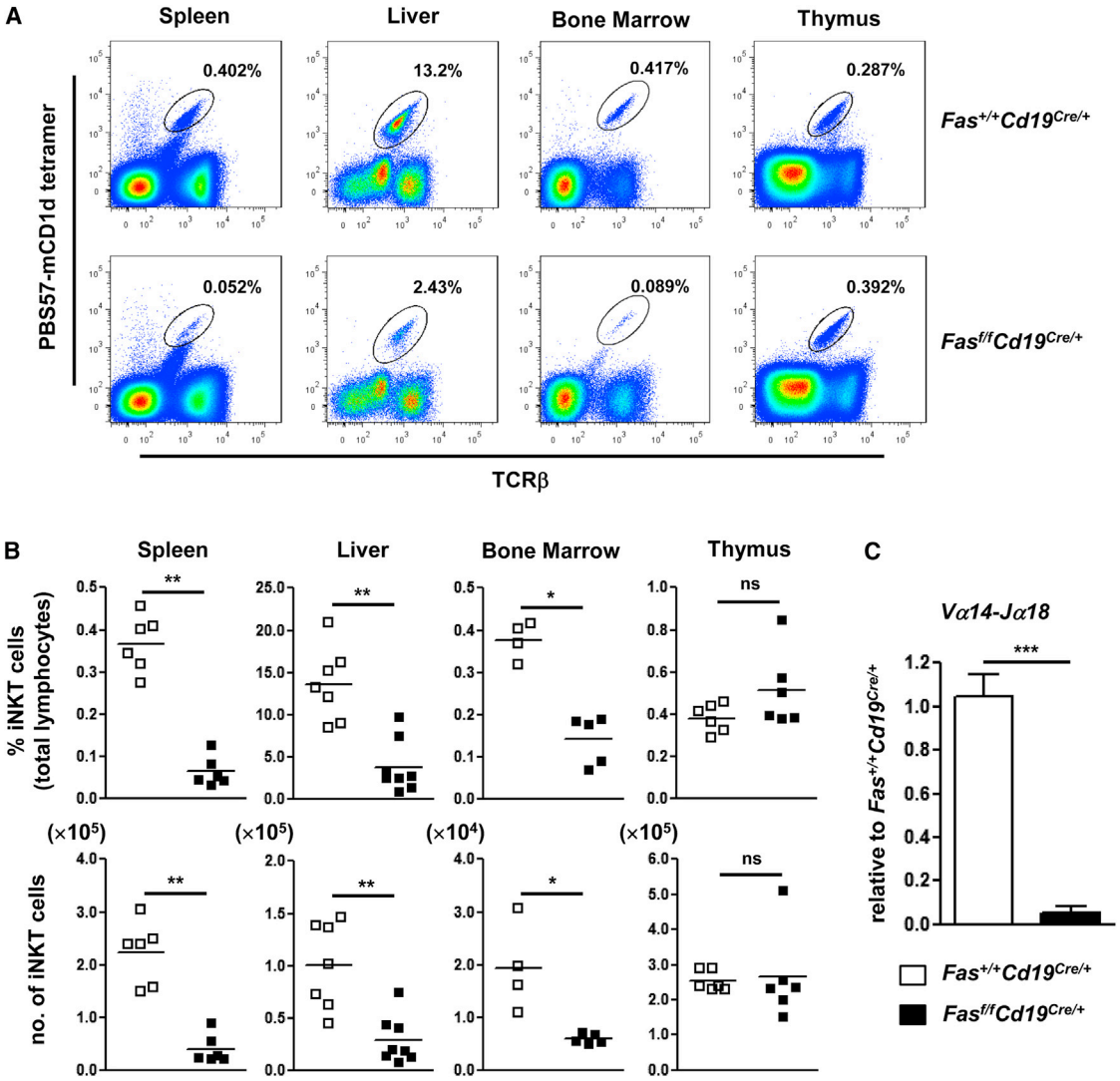

Figure 1. Reduced Peripheral iNKT Frequencies and Numbers in Autoimmune *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ Mice

(A and B) Frequencies (A) and numbers (B) of iNKT cells in spleens, livers, bone marrows and thymi of 20-week-old *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ and *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice as enumerated by flow cytometry.

(C) Rearranged *Vα14-Jα18* transcript levels in *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ and *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ splenocytes as assessed by quantitative real-time PCR.

Data in (A) are representative of more than four mice. Each symbol in (B) represents an individual mouse and small horizontal bars indicate the mean. Data in (C) are mean ± SEM of measurements obtained with four mice of each genotype. *$p < 0.01$; **$p < 0.001$; ns, not significant. See also Figure S1.

they declined in these subjects. Here, we studied autoimmune *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice lacking FAS in B cells and also found iNKT frequencies and numbers to be substantially reduced in spleens, liver and bone marrows of 20-week-old mutants compared with age-matched *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ controls (Figures 1A and 1B). This defect was also apparent in young 8-week-old *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice (Figure S1). To exclude the possibility that the observed reduction in iNKT frequencies was due to cells internalizing their invariant TCRs, we assessed the levels of rearranged splenic *Vα14-Jα18* transcripts (normalized to *Cα*) by quantitative real-time PCR. We found them to be lower in *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ compared with *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ splenocytes (Figure 1C), confirming that mutant mice indeed had fewer iNKT cells. In contrast, iNKT cell frequencies and numbers in the thymi of *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ and *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice were comparable. Thus, our data indicate that peripheral, but not thymic, iNKT cells are perturbed in autoimmune *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice.

To understand the cause of iNKT cell diminution in *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice, we examined their expression of costimulatory molecules and found higher expression of FAS, PD-1, ICOS, OX-40, FASL, CD40L, PD-L1, and PD-L2 but normal CD25 and CD28, suggesting that *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ iNKT cells were hyperactivated compared with *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ counterparts (Figure 2A). In addition, they have altered cytokine production as a greater fraction (39.5%) of iNKT cells from *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice expressed interferon-γ (IFN-γ) while a correspondingly lower fraction

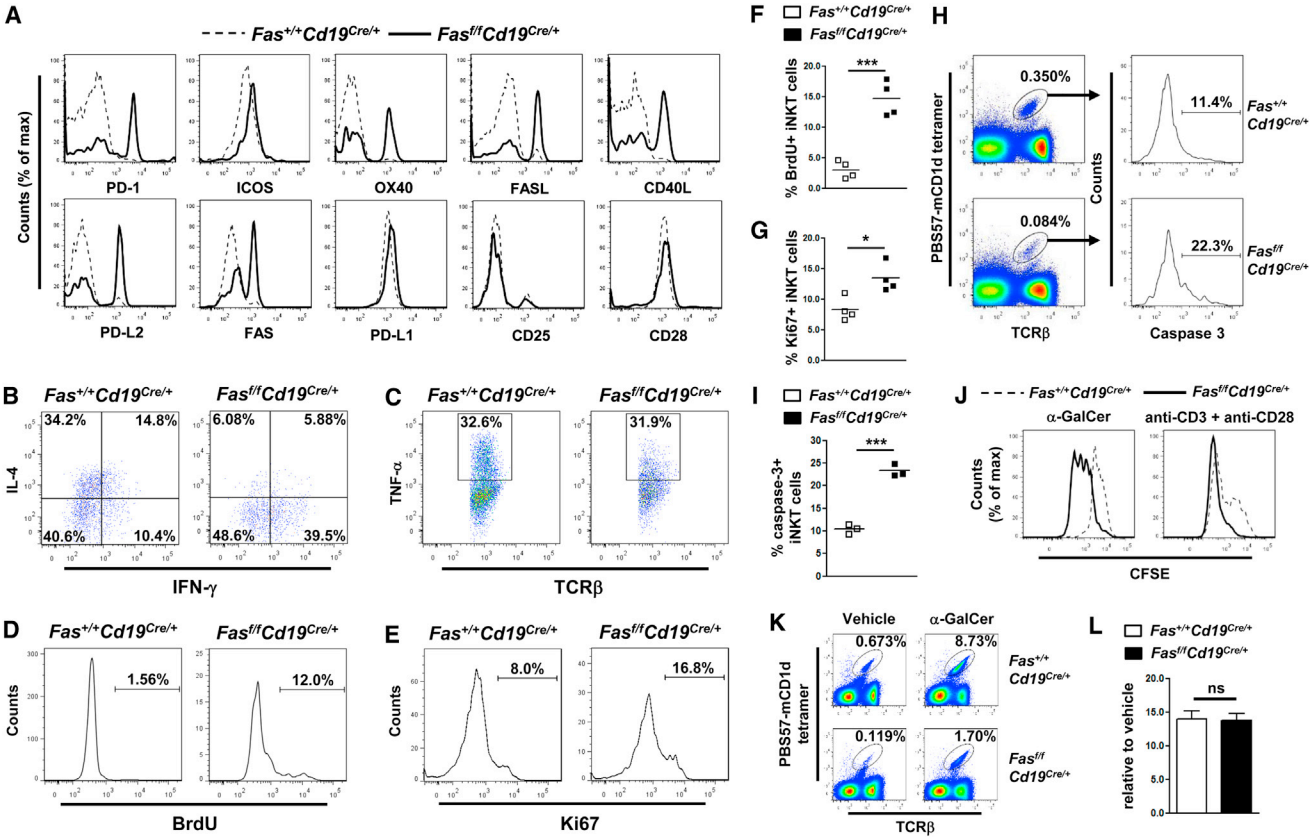

Figure 2. iNKT Cells in *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ Mice Exhibit Hyperactivation and Altered Cytokine Expression but Respond Normally to External Stimuli In Vitro and In Vivo

(A–C) Expression of cell-surface molecules on (A) and cytokine production by (B and C) splenic iNKT cells in age-matched *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ and *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice.

(D–G) Frequencies of *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ and *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ iNKT cells that incorporated BrdU 24 hr after intraperitoneal (i.p.) injection (D and F) or stained positive for Ki67 ex vivo (E and G).

(H and I) Frequencies of caspase-3-expressing iNKT cells in *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ and *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice.

(J) Proliferation of CFSE-labeled *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ and *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ iNKT cells stimulated with α-GalCer or anti-CD3 and anti-CD28 for 3 days.

(K) Splenic iNKT cell frequencies in *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ and *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice 3 days after i.p. injection with α-GalCer.

(L) Fold increase in frequency of splenic iNKT cells from *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ and *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice injected with α-GalCer with respect to mice of same genotype injected with vehicle alone.

Data in (A)–(E), (H), (J), and (K) are representative of more than three mice of each genotype. Data in (F), (G), (I), and (L) are mean ± SEM of measurements obtained with four mice. *$p < 0.01$; ***$p < 0.0001$; ns, not significant.

(6.08%) expressed IL-4 after ex vivo stimulation with phorbol-12-myristate-13-acetate (PMA) and ionomycin. In contrast, *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ iNKT cells predominantly produced IL-4 (34.2%) and less IFN-γ (10.4%; Figure 2B). However, the proportion of *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ and *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ iNKT cells producing tumor necrosis factor α (TNF-α) were similar (32.6% versus 31.9%; Figure 2C). Since iNKT cells in *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice appeared overactivated, we asked if they exhibited increased proliferation. We found iNKT cells in *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice to undergo more cell divisions as evident from the increased percentage of cells that incorporated bromodeoxyuridine (BrdU) (12.0%) compared with *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ iNKT cells (1.56%; Figures 2D and 2F). This was corroborated by the elevated frequency of iNKT cells in *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice staining positive for Ki67 (16.8% versus 8.0%; Figures 2E and 2G). Moreover, we found the proportion of iNKT cells that expressed activated caspase-3 to be 2-fold higher (22.3%) in *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ compared with *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ mice (11.4%; Figures 2H and 2I), suggesting that these cells had a greater propensity to apoptose. Collectively, the data suggest that most iNKT cells in *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice were antigenically overactivated, had altered cytokine profile, and manifested enhanced cell division and turnover.

Given the hyperactivated phenotype of iNKT cells in *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice under steady-state conditions in vivo, we investigated if they remained responsive to external stimuli in vitro and in vivo. We labeled *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ and *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ iNKT splenocytes with carboxyfluorescein diacetate succinimidyl ester (CFSE) and cultured them in the presence of α-GalCer or anti-CD3 and anti-CD28 antibodies for 3 days. Consistent with their hyperactivated phenotype, iNKT cells from *Fas*$^{f/f}$*Cd19*$^{Cre/+}$

mice underwent greater number of cell divisions compared with those from *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ mice (Figure 2J). To ascertain the relevance of these observations in vivo, we injected mice with vehicle alone or α-GalCer and sacrificed them 3 days later to enumerate splenic iNKT cell frequencies. iNKT cells from *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ mice expanded by ∼13.0-fold (0.673% in vehicle-challenged versus 8.73% in α-GalCer-injected mice; Figure 2K). iNKT cells from *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice also expanded equivalently by ∼14.3-fold (0.119% versus 1.70%). Thus, iNKT cells in mutant mice proliferated normally in response to α-GalCer (Figure 2L), suggesting that they remained functionally competent despite being numerically diminished.

As the iNKT cell defects resulted from sole dysregulation of B cells in *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice, we assessed if there were changes to B cell subset composition in these mice. We examined CD21^{hi}CD23^{int} MZ, CD21^{int}CD23^{hi} follicular (Foll), and CD21^{lo}CD23^{lo} or CD11b^{hi}CD11c^{hi} age-associated B cell (ABC) subsets. ABCs were recently identified to be autoreactive cells refractory to B cell-receptor stimulation but produced cytokines in response to innate stimuli and were abundant in aged female, but not male, subjects (Hao et al., 2011; Rubtsov et al., 2011). We found the frequency of CD21^{lo}CD23^{lo} ABCs as a fraction of B220^{+}CD93^{-} mature B cells to be significantly increased in aged *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ (23.3%) compared with age-matched *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ (4.39%) mice (Figure S2A). The frequency of Foll B cells concomitantly decreased from 86.1% in *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ to 64.4% in *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice, while MZ B cell frequency was largely unaffected. Analyses of more mice of each genotype confirmed the accrual of ABCs in *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice (Figure S2B). Total B cells in *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice were previously shown to express elevated levels of costimulatory molecules compared with *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ counterparts (Hao et al., 2008). Here, we extended the analyses to demonstrate that ABCs and not MZ or Foll B cells expressed exacerbated levels of CD80, CD86, and MHCII in *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice (Figure S2C).

Because the semi-invariant TCRs of iNKT cells recognize glycolipid antigens presented by CD1d on antigen-presenting cells, we assessed if CD1d expression on autoimmune FAS-deficient B cells was altered. Previously, human activated or memory B cells were shown to express lower levels of CD1d compared with naive or MZ-like B cells and resting B cells activated via B cell receptor or CD40L in vitro downregulated CD1d (Allan et al., 2011). It was proposed that immature CD19^{+}CD24^{hi}CD38^{hi} SLE B cells failed to support iNKT cell survival due to their reduced CD1d levels. In contrast, we found CD1d expression on *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ MZ B, Foll B, or ABCs to be largely comparable to that found on *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ counterparts (Figure S2D), ruling out changes in CD1d expression on FAS-deficient B cells as contributing to diminished iNKT frequencies in autoimmune mice (Figure 1).

We therefore hypothesized that iNKT depletion in *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice arose from overstimulation of iNKT cells by CD1d-restricted self-lipids from autoimmune B cells. If decreased CD1d expression in B cells led to iNKT diminution in autoimmune mice, one predicted consequence of removing CD1d would be to further deplete residual iNKT cells. Surprisingly, this was not the case, as we found appreciable restoration of iNKT cell frequencies and numbers in spleens, livers, and bone marrows of *Fas*$^{f/f}$*Cd1d1*$^{f/f}$*Cd19*$^{Cre/+}$ mice in which CD1d expression was ablated specifically in B cells, compared with *Fas*$^{f/f}$*Cd1d1*$^{+/+}$*Cd19*$^{Cre/+}$ mice (Figures 3A and 3B). Concomitant with the rescue of iNKT cell frequencies in *Fas*$^{f/f}$*Cd1d1*$^{f/f}$*Cd19*$^{Cre/+}$ mice, we observed substantial correction of augmented PD-1 and ICOS expression (Figure 3C) and altered cytokine production (Figure 3D) in iNKT cells. This CD1d-dependent suppression of iNKT cells by autoimmune B cells adds a different perspective to previous data showing the reverse, in which iNKT cells inhibited autoreactive B cell activation and autoantibody production triggered genetically (Yang et al., 2011) or by systemic loading of apoptotic cells (Wermeling et al., 2010). However, as there was incomplete rescue of iNKT cell numbers in *Fas*$^{f/f}$*Cd1d1*$^{f/f}$*Cd19*$^{Cre/+}$ mice, CD1d-mediated presentation of self-lipids by autoimmune B cells probably represents one of several pathways affecting iNKT cells in *Fas*$^{f/f}$*Cd1d1*$^{+/+}$*Cd19*$^{Cre/+}$ mice.

Importantly, our data suggest that CD1d-mediated self-lipid presentation by autoimmune, but not healthy, B cells actively depleted iNKT cells in *Fas*$^{f/f}$*Cd1d1*$^{+/+}$*Cd19*$^{Cre/+}$ mice, since CD1d loss in B cells of nonautoimmune *Fas*$^{+/+}$*Cd1d1*$^{f/f}$*Cd19*$^{Cre/+}$ mice (Figure S3A) did not affect iNKT frequencies and numbers in various organs (Figure S3B). These results suggest that CD1d-mediated presentation of self-lipids is essential for iNKT maturation in the thymus (Gapin et al., 2001) of normal mice and also impacts peripheral iNKT homeostasis in autoimmune mice.

We next examined if FAS-deficient ABCs could deplete normal iNKT cells in a CD1d-dependent manner in vitro. We cocultured 2 × 10^{6} ABCs enriched from spleens of *Fas*$^{+/+}$*Cd1d1*$^{+/+}$*Cd19*$^{Cre/+}$, *Fas*$^{f/f}$*Cd1d1*$^{+/+}$*Cd19*$^{Cre/+}$ or *Fas*$^{f/f}$*Cd1d1*$^{f/f}$*Cd19*$^{Cre/+}$ mice with 2 × 10^{5} CFSE-labeled wild-type iNKT cells enriched from thymi of C57BL/6 mice for 3 days. The frequency of iNKT cells was decreased from 3.22% when they were cocultured with FAS-sufficient ABCs to 0.672% when they were incubated with FAS-deficient ABCs (Figure 3E). Importantly, iNKT cell frequency remained largely unaffected (2.68%) when they were cocultured with FAS-deficient ABCs that also lack CD1d. It is possible that the expansion of and hence increase in frequency of FAS-deficient ABCs led to proportional reduction in iNKT frequency. However, enumeration of cells in cocultures indicated that the number of iNKT cells was significantly lower when they were cultured with FAS-deficient compared with FAS-sufficient ABCs and unperturbed when cultured with FAS-deficient ABCs that also lacked CD1d (Figure 3F). In addition, iNKT cells proliferated robustly when cocultured with FAS-deficient ABCs but weakly when cocultured with FAS-sufficient ABCs or ABCs deficient in both FAS and CD1d (Figure 3G). Similar observations were made with coculture of 2 × 10^{5} ABCs and 2 × 10^{5} iNKT cells. Although the frequency of iNKT cells was similar whether in the presence of FAS-sufficient or deficient ABCs (Figure S4A), the number of iNKT cells was significantly reduced with FAS-deficient ABCs but unchanged with ABCs lacking FAS and CD1d (Figure S4B). In the absence of overt lipid agonists, our data suggest that FAS-deficient ABCs most likely activated iNKT cells via CD1d-mediated presentation of endogenous lipids. Altered expression of PD-1, ICOS, and cytokines observed in iNKT cells cocultured with FAS-deficient ABCs reverted to normalcy when ABCs doubly deficient in FAS and

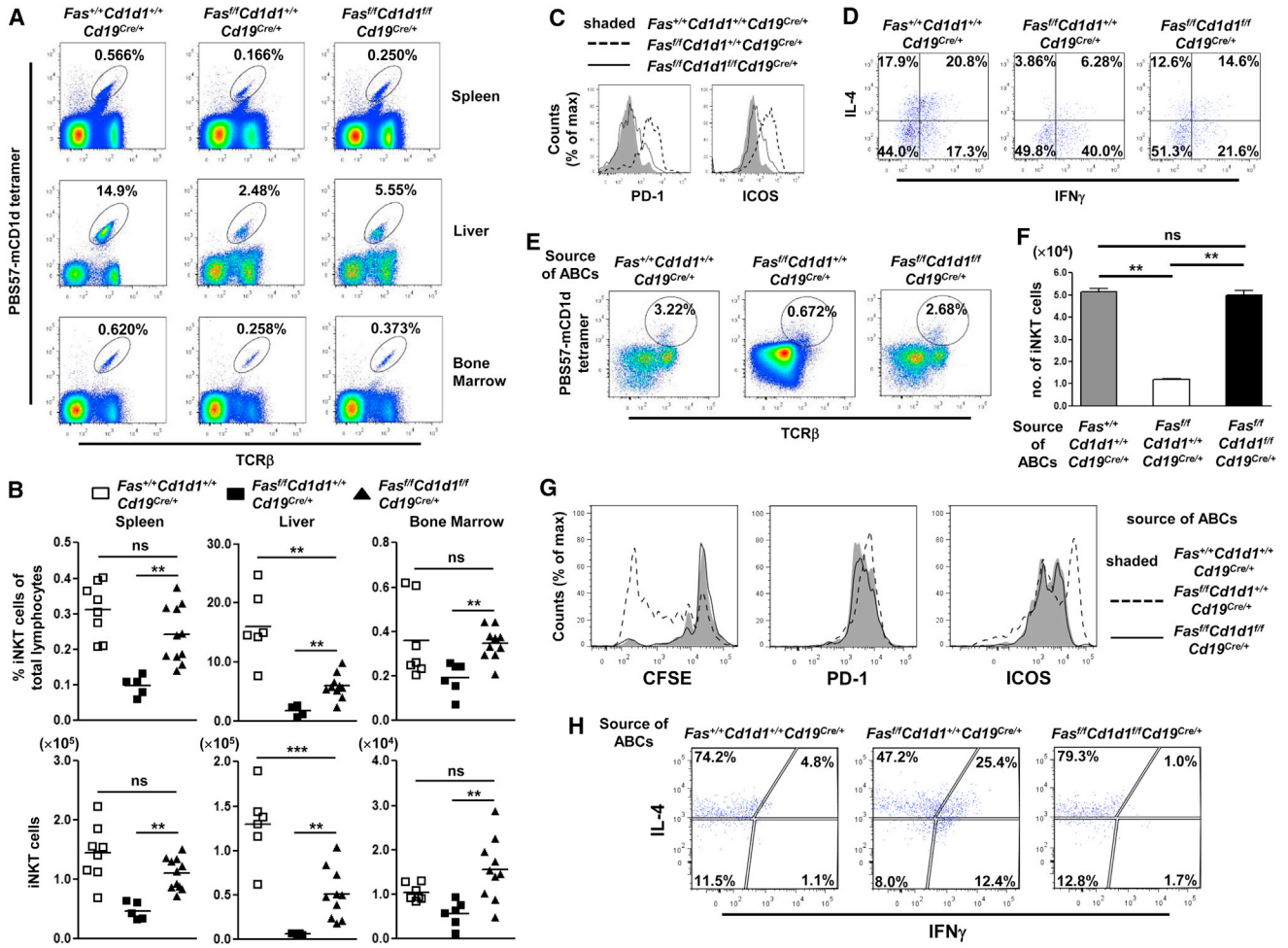

Figure 3. CD1d-Mediated Presentation of Self-Lipids by Autoimmune B Cells Hyperactivates and Depletes iNKT Cells In Vivo and In Vitro

(A and B) Frequencies (A) and numbers (B) of iNKT cells in spleens, livers, and bone marrows of age-matched *Fas*$^{+/+}$*Cd1d1*$^{+/+}$*Cd19*$^{Cre/+}$, *Fas*$^{f/f}$*Cd1d1*$^{+/+}$*Cd19*$^{Cre/+}$, and *Fas*$^{f/f}$*Cd1d1*$^{f/f}$*Cd19*$^{Cre/+}$ mice.

(C and D) Expression of ICOS and PD-1 on (C) and cytokine production by (D) iNKT cells from various mice as in (A).

(E–H) In vitro coculture of 2 × 10^6 splenic ABCs from mice as in (A) with 2 × 10^5 iNKT cells from thymi of C57BL/6 mice for 3 days. Shown are frequencies (E), numbers (F), and proliferation and expression of ICOS and PD-1 of (G) and cytokines produced by (H) iNKT cells.

Data in (B) and (F) are mean ± SEM of measurements obtained with four or more mice of each genotype. The rest of data are representative of four or more mice analyzed. **p < 0.001; ***p < 0.0001; ns, not significant. See also Figures S2–S4.

CD1d were employed (Figures 3G and 3H). Together, the data strongly suggest that autoimmune, but not normal, CD1d$^+$ B cells present self-lipids via CD1d that hyperactivated and severely depleted peripheral iNKT cells.

Based on these results and previous work demonstrating the ability of self-lipids to activate iNKT cells during host immunity against pathogens (Brennan et al., 2011; Zeissig et al., 2012), we postulated that altered expression of certain self-lipids in FAS-deficient B cells, when presented in the context of CD1d, potentially activated and promoted the apoptosis of iNKT cells. We hence examined endogenous lipids in splenic B cells from *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ and *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice using liquid chromatography-mass spectrometry (LC-MS). Profiling of lipid metabolites revealed changes in the lipidome of autoimmune compared with normal B cells. Expression levels of selected globotrihexosylceramides (Gb3 [d34:1], Gb3 [d42:2], and Gb3 [d42:1]) and phosphatidylglycerol (PG [32:0]) were increased while that of cholesterol sulfate was decreased in FAS-deficient compared with FAS-sufficient B cells (Figure 4A). On the other hand, the ceramide (Cer [d42:2]) level was unchanged. The molecular identities of these lipids were verified using MS^2 fragmentation analysis, and Figure 4B shows the example of Gb3 (d42:2), in which its decomposition yielded the expected daughter moieties. We further validated the LC-MS results for Gb3 by using a specific antibody to detect its surface expression on various B cell subsets from *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ and *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice. We found cell-surface Gb3 expression to be confined primarily to ABCs with little being detected in MZ or Foll B cells. Interestingly, FAS-deficient ABCs expressed Gb3 at levels substantially higher than FAS-sufficient cells (Figure 4C). This

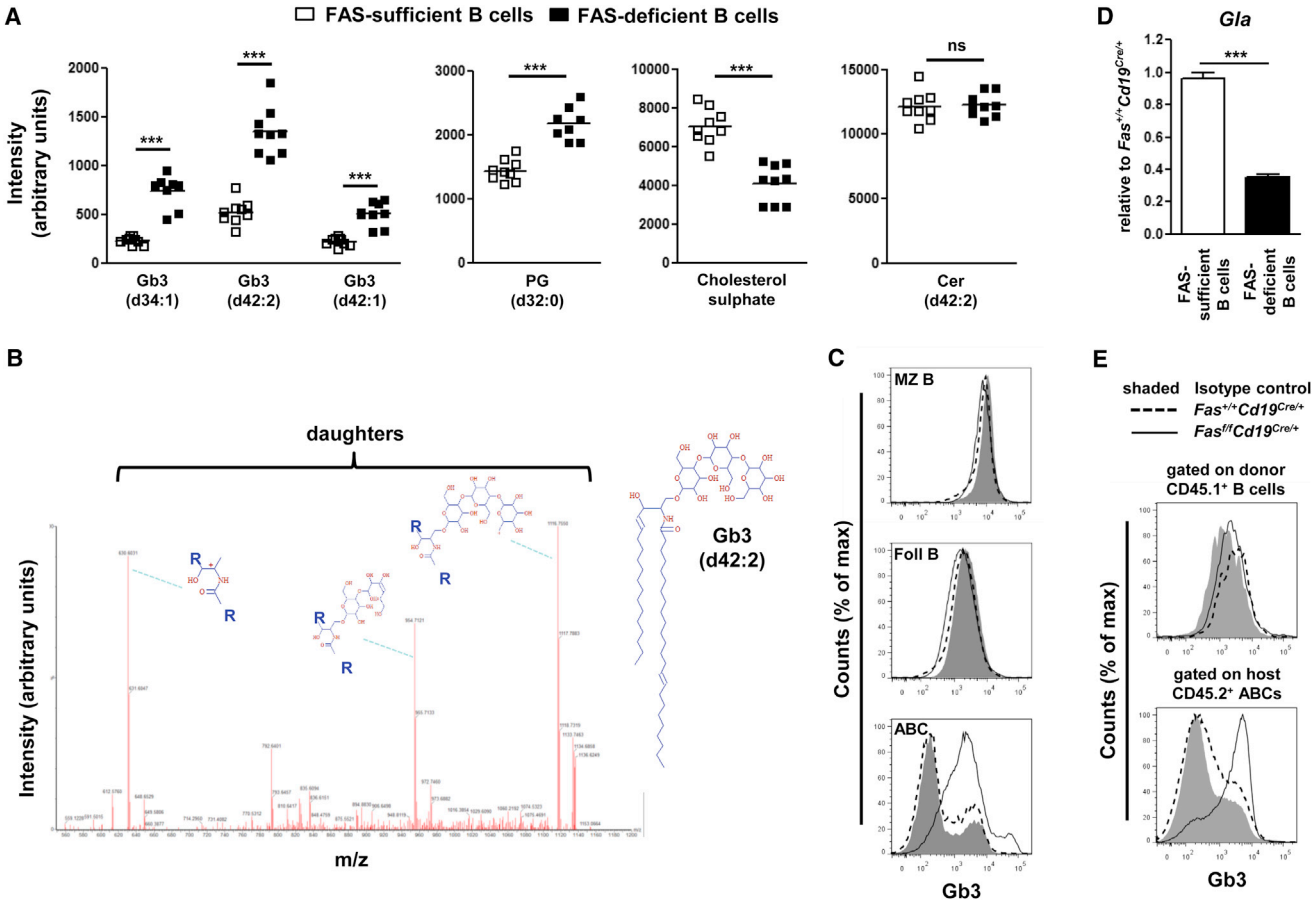

Figure 4. Autoimmune FAS-Deficient B Cells Have Altered Lipidome

(A) Intensities of Gb3s (d34:1, d42:2 and d42:1), PG (d32:0), cholesterol sulfate, and ceramide (Cer [d42:2]) in FAS-sufficient and FAS-deficient splenic B cells as measured by LC-MS.

(B) MS^2 spectrum of one of the Gb3s identified in FAS-deficient B cells depicting protonated Gb3 (d42:2) peak (1,134.789 m/z). Fragmentation analysis indicated all major daughter species were accounted for by stepwise cleavage of three sugar residues. The full molecular structure of Gb3 (d42:2) is shown (right).

(C) Gb3 expression on splenic B cell subsets from $Fas^{+/+}Cd19^{Cre/+}$ and $Fas^{f/f}Cd19^{Cre/+}$ mice. Shaded histograms depict staining by isotype control antibody.

(D) *Gla* transcript levels in FAS-deficient compared with FAS-sufficient ABCs.

(E) Gb3 expression on gated donor $CD45.1^+$ B cells (top) and host $CD45.2^+$ ABCs (bottom) in $Fas^{+/+}Cd19^{Cre/+}$ or $Fas^{f/f}Cd19^{Cre/+}$ mice 10 days after intravenous injection of 10×10^6 $CD45.1^+$ B6.SJL splenocytes.

Each symbol in (A) represents a single mouse sample, and small horizontal bars indicate the mean. Data in (C) and (E) are representative of three mice of each genotype. Data in (D) are mean ± SEM of measurements obtained with three mice. ***$p < 0.0001$; ns, not significant.

complements recent work showing elevated Gb3 in SLE T cells (McDonald et al., 2014).

A previous study showed lysosomal α-galactosidase A (α-galA) controlled the generation of self-lipids, and iNKT cells were numerically reduced in $Gla^{-/-}$ mice lacking α-galA (Darmoise et al., 2010). We found $Fas^{f/f}Cd19^{Cre/+}$ mice to phenocopy $Gla^{-/-}$ mice in the reduction of iNKT cells. Interestingly, we also found FAS-deficient ABCs to express significantly reduced levels of *Gla* transcript compared with FAS-sufficient ABCs (Figure 4D), further supporting the hypothesis that autoimmune B cells, with reduced *Gla* expression, accumulated altered self-lipids that overactivated and depleted iNKT cells in $Fas^{f/f}Cd19^{Cre/+}$ mice in a CD1d-dependent manner.

To ascertain if enhanced Gb3 expression as a surrogate biomarker of the altered lipidome of FAS-deficient ABCs is cell intrinsic or an effect of the autoimmune microenvironment, we adoptively transferred congenically distinguished $CD45.1^+$ splenocytes from B6.SJL mice into $Fas^{+/+}Cd19^{Cre/+}$ or $Fas^{f/f}Cd19^{Cre/+}$ mice expressing CD45.2. Recipient mice were analyzed at day 10 for Gb3 expression on donor $CD45.1^+$ B cells and host $CD45.2^+$ ABCs. Donor B cells, whether transferred into $Fas^{+/+}Cd19^{Cre/+}$ or $Fas^{f/f}Cd19^{Cre/+}$ mice, expressed comparable levels of cell-surface Gb3 (Figure 4E, top), suggesting that Gb3 accumulation in FAS-deficient ABCs is unlikely due to the autoimmune environment in $Fas^{f/f}Cd19^{Cre/+}$ mice. As before, Gb3 was increased in $CD45.2^+$ FAS-deficient ABCs compared with FAS-sufficient cells (Figure 4E, bottom).

In summary, we show that iNKT cell numbers were reduced in autoimmune mice with sole dysregulation of B cells. FAS-deficient B cells likely presented self-lipids via CD1d that overstimulated

and depleted iNKT cells. Lipidomic profiling revealed alterations in certain endogenous lipids in FAS-deficient B cells. Future work is required to determine the identity of "autoimmune" self-lipids and whether similar lipidome alterations are found in B cells rendered autoimmune by other genetic lesions. Our data illustrate for that the lipidome of autoimmune B cells is altered in comparison to normal B cells and has adverse effects on peripheral iNKT cells. The findings raise the intriguing possibility that the metabolome of B cells could impact the homeostasis of other immune cell types and add to growing evidence of additional B cell function beyond antibody production.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 (CD45.2), B6.SJL (CD45.1), *Fas*$^{f/f}$ (Hao et al., 2004), *Cd1d1*$^{f/f}$ (Bai et al., 2012), and *Cd19*$^{Cre/+}$ (Rickert et al., 1995) mice were from The Jackson Laboratory and bred in our animal facilities under specific-pathogen-free conditions. Experiments with mice were conducted according to guidelines issued by A*STAR Biological Resource Centre Institutional Animal Care and Use Committee.

Cell Suspensions and Flow Cytometry

Spleen, thymus, liver mononuclear, and bone marrow cells were prepared by standard methods. Before labeling with relevant fluorochrome-conjugated antibodies, cells were treated with F_c block. iNKT cells were stained with allophycocyanin (APC)-conjugated CD1d-PBS57 tetramer and other relevant cell-surface markers. Antibodies against TCRβ (H57-597), B220 (RA3-6B2), CD21/CD35 (7E9), CD23 (B3B4), CD38 (90), CD93 (AA4.1), CD274 (B7-H1/PD-L1; 10F.9G2), CD278 (ICOS; C398.4A), CD279 (PD-1; 29F.1A12), CD45.1 (A20), CD45.2 (104), phycoerythrin (PE)-Cy7-conjugated streptavidin, and rat immunoglobulin M (IgM) isotype control antibody (RTK2118) were from BioLegend. Antibodies against CD69 (H1.2F3), GL7 (GL7), CD28 (37.51), CD134 (OX40; OX-86), and CD273 (B7-DC/PD-L2; TY25) were from eBioscience. Antibodies against CD25 (7D4), CD95 (Fas/APO-1; Jo2), CD154 (CD40L; MR1), CD178 (FasL; MFL4), and Ki67 (B56) were from BD Pharmingen. Active caspase-3 expression was visualized using the CaspGLOW Staining Kit (BioVision). Purified anti-Gb3 (CD77; 38-13) and goat anti-rat IgM were from GenTex. Samples were acquired on an LSRII cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Intracellular Cytokine Production

A total of 5 × 10^6 splenocytes were stimulated with 50 ng/ml PMA and 0.5 μg/ml ionomycin (Sigma-Aldrich) in the presence of brefeldin A at 37°C for 5 hr. After staining with surface markers, cells were fixed and permeabilized using the Cytofix/CytoPerm Kit (BD Pharmingen) and stained with antibodies against IFN-γ (XMG1.2; eBioscience), anti-IL-4 (11B11; BD Pharmingen), or anti-TNF-α (MP6-XT22; eBioscience).

Splenic iNKT Cell Stimulation

For in vivo studies, mice were injected intraperitoneally with 1 mg BrdU (Sigma-Aldrich) 24 hr before analysis. Splenic iNKT cells were stained with antibodies against surface markers and processed with FITC BrdU Flow Kit (BD Pharmingen) before flow cytometric analysis. Mice were also injected with 100 μg/kg of α-GalCer and sacrificed 3 days later to examine splenic iNKT cell numbers (Harada et al., 2004). For in vitro studies, splenocytes were labeled with 2 μM CFSE (Invitrogen) and iNKT cells enriched using mouse NK1.1+ iNKT Cell Isolation Kit (Miltenyi Biotec). α-GalCer (BioVision) was heated at 80°C and sonicated for 10 min. A total of 1 × 10^5 splenic iNKT cells were then incubated in the presence of 100 ng/ml sonicated α-GalCer or 1 μg/ml plate-bound anti-CD3 and 2 μg/ml soluble anti-CD28 for 3 days.

Coculture of ABCs with iNKT Cells

ABCs were enriched from the spleens of *Fas*$^{+/+}$*Cd1d1*$^{+/+}$*Cd19*$^{Cre/+}$, *Fas*$^{f/f}$*Cd1d1*$^{+/+}$*Cd19*$^{Cre/+}$, and *Fas*$^{f/f}$*Cd1d1*$^{f/f}$*Cd19*$^{Cre/+}$ mice using PE-conjugated antibodies against CD21/CD35 (7E9), CD23 (B3B4), and CD43 (S11) (all from BioLegend) and anti-PE microbeads. iNKT cells were enriched from thymi of C57BL/6 mice using APC-conjugated CD1d-PBS57 tetramer and anti-APC microbeads. ABCs were cocultured with CFSE-labeled C57BL/6 iNKT cells for 3 days.

Adoptive Transfer of CD45.1$^+$ Splenocytes

A tota of 10 × 10^6 splenocytes from B6.SJL (CD45.1$^+$) mice were injected into CD45.2$^+$ *Fas*$^{+/+}$*Cd19*$^{Cre/+}$ and *Fas*$^{f/f}$*Cd19*$^{Cre/+}$ mice via their lateral tail veins. Recipient mice sacrificed 10 days after injection were analyzed for splenic Gb3 expression on donor CD45.1$^+$ B cells and host CD45.2$^+$ ABCs.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated using TRIzol (Invitrogen), precipitated with isopropanol, and cDNA prepared with RevertAid H Minus First-Strand cDNA Synthesis Kit (Fermentas) using oligo(dT)$_{12–18}$ as primer. SYBR green Master Mix (Applied Biosystems) was used for real-time PCR. Primer sequences were *Vα14* forward, 5′-GTC CTC AGT CCC TGG TTG TC-3′; *Jα18* reverse, 5′-CAA AAT GCA GCC TCC CTA AG-3′; *Gla* forward, 5′-GAC ATT GAT GCG CAG ACA TTT GA-3′; *Gla* reverse, 5′-TTC GGC CTG TCC TGT TCA AG-3′; *Cα* forward, 5′-CCT CTG CCT GTT CAC CGA CTT-3′; *Cα* reverse, 5′-CAG TCA ACG TGG CAT CAC A-3′; *Actb* forward, 5′-CCG CGA GCA CAG CTT CTT TG-3′; *Actb* reverse, 5′-ACA TGC CGG AGC CGT TGT C-3′. The mRNA levels of gene transcripts were normalized to those of *Cα* or *Actb* (β-actin).

Lipid Extraction and Lipid Profiling with LC-MS

B cells were enriched from the spleens of mice using anti-CD19-conjugated microbeads (Miltenyi Biotec). After centrifugation, B cell pellets were lysed in ice-cold methanol:tricine:chloroform mixture. The chloroform layer containing lipids was collected and stored at −80°C. The chloroform was blown dry with liquid nitrogen and the lipid extract reconstituted in LC solvents. An ultraperformance LC Acquity system (Waters Corporation) coupled to a high-resolution mass spectrometer, Xevo Q-TOF (Waters Corporation), was used for intracellular lipid profiling. The LC-MS data preprocessing was described previously (Chong et al., 2009; see the Supplemental Experimental Procedures for details of LC setup parameters). Masses of shortlisted peaks were compared against entries in the Kyoto Encyclopedia of Genes and Genome and the Human Metabolome Database. Peaks with matches within a 10 ppm mass accuracy window were assigned putative identities, some of which were verified by matching observed MS^2 fragmentation patterns with theoretical fragments generated by Mass Frontier 5.1 software (HighChem) as standards were not commercially available.

Statistical Analyses

Differences in values between samples were compared by Student's t test with Welch's correction using Prism (GraphPad Software) except for data in Figures 1B, 3B, S1B, and S3B, to which the Mann-Whitney test was applied. Values of $p \leq 0.05$ were regarded as statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.08.043.

AUTHOR CONTRIBUTIONS

A.H.-M.T. and K.-P.L. conceived and designed the study. A.H.-M.T., W.P.-K.C., S.-W.N., N.B., and S.X. performed experiments. A.H.-M.T., W.P.-K.C., and K.-P.L. analyzed data. A.H.-M.T. and K.-P.L. wrote the manuscript.

ACKNOWLEDGMENTS

We thank G. Zeng, F. Ng, and A. Sanny for technical assistance; the staff of the Biological Resource Centre for care and maintenance of mice; and members of the laboratory for discussions. The US NIH Tetramer Core Facility provided the APC-conjugated CD1d-PBS57 tetramer. This study was supported by the

Biomedical Research Council of the Singapore Agency for Science, Technology and Research.

Received: February 19, 2014
Revised: June 4, 2014
Accepted: August 20, 2014
Published: September 25, 2014

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